



# Nuclear factor kappa B in patients with a history of unstable angina: case re-opened

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## Abstract

This study aims at assessing NF- $\kappa$ B activity in unstable angina (UA) patients free of symptoms after a 1 year follow-up (1YFU). Plasma oxidized low-density lipoproteins (oxLDL), circulating NF- $\kappa$ B, Interleukin 6 (IL-6) and Interleukin 1 $\beta$  (IL-1 $\beta$ ), high-sensitivity C-reactive protein (hs-CRP), as markers of oxidative stress and inflammation and plasma double-stranded DNA (ds-DNA), as marker of Neutrophil Extracellular Traps (NETs), were measured in 23 of the previously enrolled 27 UA patients. These measurements were compared to the UA data at baseline, and then compared to the data derived from the stable angina (SA) and controls (C) enrolled in our previous study (we demonstrated that UA had higher levels of NF- $\kappa$ B compared to SA and C). After a 1YFU, UA patients show a significant decrease in NF- $\kappa$ B, IL-6, hs-CRP, oxLDL, and ds-DNA plasma levels ( $p < 0.001$ ) and in IL-1 $\beta$  and White Blood Cells (WBC) ( $p < 0.005$ ), without differences in lipid and glucose assessment. If compared to SA and C, UA after a 1YFU have higher levels of NF- $\kappa$ B, IL-6, ds-DNA, WBC, and oxLDL compared to C ( $p < 0.001$ ), but only IL-6 is higher than SA ( $p < 0.001$ ). No differences are found in lipid and glucose assessment. After a 1YFU, patients with a history of UA improve their oxidative and inflammatory status, such as the levels of circulating ds-DNA, without achieving the status of C. They become comparable to SA subjects. This study provides new insight on the multiple and apparently contradictory facets of NF- $\kappa$ B in UA and on its possible role as mediator in NETs' formation.

**Keywords** Unstable and stable angina · Nuclear factor kappa B · Inflammation · Double-stranded DNA · NETosis

## Introduction

Coronary artery disease is a common complex atherosclerotic pathology associated with substantial morbidity and mortality [1]. Unstable angina (UA) is defined as myocardial ischemia at rest or minimal exertion in the absence of cardiomyocyte necrosis [2]. Compared with non-ST-elevation myocardial infarction patients, individuals with UA do not experience myocardial necrosis, have a substantially lower risk of death, and appear to derive less benefit from

intensified anti-platelet therapy as well as early invasive strategy [3, 4]. UA prevalence is expected to be 10% [5] among unselected patients admitted with acute chest pain to the emergency department (ED).

Nuclear factor kappa B (NF- $\kappa$ B) is the central regulator of innate and adaptive response with hundreds of target genes, some with pro-inflammatory effects, and some promoting cell survival [6]. NF- $\kappa$ B intervenes in the transcription of a large number of inflammatory genes coding for cytokines, chemokines, and adhesion molecules [7]. NF- $\kappa$ B can be activated via the canonical and the non-canonical pathway [7]. NF- $\kappa$ B is normally held in the cytoplasm in complex with the inhibitor- $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ). Canonical activation of NF- $\kappa$ B involves phosphorylation of I $\kappa$ B $\alpha$  and its proteasome degradation when inflammation occurs [8]. This pathway is mainly activated in response to pro-inflammatory stimuli. Non-canonical NF- $\kappa$ B signalling is important for the development and maintenance of primary and secondary lymphoid organs, and adaptive immune responses [7, 8].

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A key role for NF- $\kappa$ B is essential in the pathophysiology of myocardial re-perfusion injury, ischemic preconditioning, and UA [9].

Nevertheless, the protective role for NF- $\kappa$ B during pathological remodelling of the heart is a source of controversy [9]. This is due to the evidence that NF- $\kappa$ B also regulates many different anti-apoptotic factors, such as cellular inhibitors of apoptosis, caspase inhibitors, and Bcl-2 family members [10].

Cell-free DNA (cf-DNA) is present in small amounts in plasma of healthy subjects [11], but it has been reported to be elevated in various clinical disorders [12]. It has been correlated with the degree of tissue damage, originating from necrosis and apoptosis of blood and tissue cells [12]. In particular, cf-DNA has been found to be elevated in patients with acute coronary syndrome in different studies [13, 14] with a prognostic potential [15].

Part of cf-DNA is double-stranded DNA (ds-DNA). Ds-DNA is a marker of the peculiar process through which neutrophils and other cell types expel ds-DNA [16, 17]. A fascinating novel explanation of how DNA can actively be released under inflammatory conditions has recently been discovered [16, 17]. It is an evolutionary and highly conserved first-line defence mechanism that allows neutrophils to expel their DNA, forming a meshwork of chromatin and proteins termed neutrophil extracellular traps (NETs) [16, 17]. NETs are the results of a peculiar form of cell death that is morphologically characterized by the loss of intracellular membranes before the integrity of the plasma membrane can be lost. The death of neutrophils with NETs formation is called NETosis. The main function of NETs is trapping and killing pathogens. Nevertheless, recent studies suggest NETs interference in other diseases (venous thromboembolism, cancer, autoimmune diseases [18–22]) and in atherosclerosis progression [23–26], as recently reviewed [27].

We have previously demonstrated [28] that UA patients have higher levels of NF- $\kappa$ B compared to stable angina (SA) patients, and both have higher levels compared to coronary artery disease-free controls (C). The activation of NF- $\kappa$ B in circulating cells of UA patients is, at least in part, induced by oxidized low-density lipoproteins (oxLDL).

The purpose of the present study is to assess NF- $\kappa$ B activity and related-circulating molecules in the same UA patients now free of symptoms after a 1 year follow-up (1YFU).

This study aims also to provide new insight on the multiple and apparently contradictory facets of NF- $\kappa$ B in UA, discussing its deleterious but also its less known survival-promoting effects. Moreover, a new field of research is proposed concerning the possible role of NF- $\kappa$ B as mediator in NETs formation.

## Materials and methods

### Ethical considerations

The study was conducted in accordance with the ethical standards laid down in the Helsinki Declaration of 1975 and its late amendments. All participants provided written consent prior to commencing the study and the local ethical committee (University of Verona-Azienda Ospedaliera Universitaria Integrata Verona) approved the study.

### Recruitment of participants

The study population and the measurement methods of NF- $\kappa$ B activity and related-circulating molecules have been previously described [28]. In addition, exclusion criteria were maintained as explained in [28].

As described [28], UA were patients with at least two episodes of rest anginal pain or one episode lasting more than 20 min in the previous 48 h preferably, but not necessarily, associated with electrocardiographic modifications (T-wave inversion, ST-segment depression, and transient ST-segment elevation) and a normal value of I-troponin on admission and during the first 24 h.

All 27 previously enrolled UA patients were recalled after 1 year and a blood sample collection was proposed.

Blood sample collection, peripheral blood mononuclear cells (PBMC) isolation, oxidized low-density lipoproteins (oxLDL) plasma levels, circulating NF- $\kappa$ B, high-sensitivity C-Reactive Protein (hs-CRP), lipid assessment, and white blood cells (WBC) were evaluated as previously described [28]. Circulating Interleukin (IL)-6 and 1- $\beta$  were tested according to the methods described in our previous study [29].

Fluorescent assay Quant-iT™ PicoGreen® ds-DNA Reagent and Kits (Invitrogen) [30] has been used to measure ds-DNA in serum of UA, SA, C, and UA 1YFU patients.

### Statistical analysis

Data were summarized as mean  $\pm$  standard deviation or median (first quartile; third quartile) for normally and non-normally distributed variables, respectively. Differences values were tested using a paired-sample Student's *t* test or Wilcoxon matched-pair signed-rank test, accordingly to the type of distribution. Statistical analyses were performed with STATA 14.1 and a 0.05 significance level was adopted.

## Results

Twenty-three of the twenty-seven previously enrolled UA patients accepted the 1YFU-recall.

Two patients (males) were excluded for recent coronary artery by-pass grafting, one patient (male) was excluded for malignancy onset, and one patient (male) denied his consent to the 1YFU evaluation. Then, the study setting was composed of 23 UA patients after 1YFU (UA1YFU): 3 females and 20 males now free of symptoms related to angina.

Baseline clinical characteristics of the patients are listed in Table 1 in [28] and [29].

Nevertheless, a summary table modified from this has been re-created: Table 1. Drug therapy was now similar in the UA1YFU group: acetylsalicylic acid, angiotensin-converting enzyme inhibitor,  $\beta$ -blocker, and statin.

Table 2 depicts the distribution of concentrations of activated circulating NF- $\kappa$ B in PBMC, serum ds-DNA, circulating cytokines, OxLDL, lipid and glucose assessment, and hs-CRP and WBC levels of UA patients at baseline and after a 1YFU. No significant differences in platelets count or in Mean Platelet Volume (MPV) are found ( $210.000 \pm 5000$   $10^9/L$  and 91 fL for UA at baseline versus  $200.000 \pm 4000$   $10^9/L$  and 93 fL after 1YFU,  $p$  0.03).

NF- $\kappa$ B, IL-6, hs-CRP, oxLDL, and ds-DNA levels are significantly lower in UA patients after 1YFU, compared to the year before ( $p < 0.001$ ).

**Table 1** Baseline clinical characteristics of the groups of patients (modified from Table 1 of [28])

	C ( $n=27$ )	SA ( $n=29$ )	UA ( $n=27$ )	$p$ value	UA1YFU ( $n=23$ )	$p$ value
Age (years)	$66 \pm 11$	$61 \pm 9$	$61 \pm 9$	NS	$62 \pm 6$	NS
Family history	37%	66%	38%	NS	37%	/
Smoke	18%	28%	46%	NS	<b>0% none</b>	<b>&lt; 0.01</b>
Hypercholesterolemia	41%	79%	69%	< 0.05	68%	/
Hypertension	48%	79%	61%	NS	60%	/
Diabetes	7%	14%	15%	NS	15%	/
ACE-I	63%	59%	35%	NS	<b>100%</b>	<b>&lt; 0.01</b>
Statins	11%	24%	11%	NS	<b>100%</b>	<b>&lt; 0.01</b>
Aspirin	30%	76%	50%	< 0.05	<b>100%</b>	<b>&lt; 0.01</b>
Previous ACS	None	65%	31%	< 0.01	30%	/
Previous PCI	None	45%	8%	< 0.01	7%	/

Data are expressed in percent

C controls, UA unstable angina patients, UA1YFU unstable angina patients after a 1 year follow-up, ACE-I angiotensin-converting enzyme inhibitors, ACS acute coronary syndrome, PCI percutaneous coronary intervention, NS not significantly different, / not re-calculated

**Table 2** Laboratory data of UA patients at baseline and after a 1YFU

		UA baseline ( $n=23$ )	UA1YFU ( $n=23$ )	$p$ value
NF- $\kappa$ B	ng/ $\mu$ g cell protein	1.52 (1.54; 1.72)	0.94 (0.35; 1.2)	<b>&lt; 0.001</b>
IL-6	pg/mL	$6.93 \pm 3.43$	$3.03 \pm 1.91$	<b>&lt; 0.001</b>
IL-1 $\beta$	pg/mL	0.79 (0.17; 1.37)	0.15 (0.01; 0.28)	<b>0.016</b>
hs-CRP	mg/dL	0.98 (0.08; 1.21)	0.42 (0.26; 0.53)	<b>&lt; 0.001</b>
oxLDL	$\mu$ g/mL	$38.7 \pm 4.39$	$23.1 \pm 3.54$	<b>&lt; 0.001</b>
LDL	mg/dL	$140 \pm 26$	$128 \pm 27$	0.264
HDL	mg/dL	$44.3 \pm 3.6$	$48.3 \pm 11.2$	0.145
Cholesterol	mg/dL	$215 \pm 39$	$202 \pm 33$	0.302
Triglycerides	mg/dL	$149 \pm 54$	$160 \pm 50$	<b>0.819</b>
Glucose	mg/dL	$101 \pm 15$	$103 \pm 46$	0.361
WBC count	$10^9/L$	$9.53 \pm 1.8$	$7.30 \pm 1$	0.002
ds-DNA	ng/mL	$23 \pm 1$	$14 \pm 1$	<b>&lt; 0.001</b>

Normally distributed continuous variables are expressed as mean  $\pm$  standard deviation, while non-normally distributed variables are presented as median and interquartile range

UA unstable angina patients, UA1YFU unstable angina patients after a 1 year follow-up, NF- $\kappa$ B nuclear factor kappa B, IL-6 Interleukin 6, IL-1 $\beta$  Interleukin 1 $\beta$ , hs-CRP high-sensitivity C-Reactive protein, oxLDL oxidized low-density lipoprotein, LDL low-density lipoprotein, HDL high-density lipoprotein, WBC white blood cells, ds-DNA double-stranded DNA

IL-1 $\beta$  and WBC levels are significantly lower in UA patients after 1YFU compared to the year before ( $p < 0.005$ ). No significant differences are found in routine lipid and glucose assessment.

Figures 1, 2 show, respectively, the levels of NF-kB (expressed in ng/ $\mu$ g cell protein) and ds-DNA (expressed in ng/mL) in UA patients at baseline and after a 1YFU, compared with SA patients and C.

NF-kB (Fig. 1) and ds-DNA (Fig. 2) levels in UA1YFU are significantly lower compared to UA baseline ( $p < 0.001$ ) and not significantly different compared to SA.

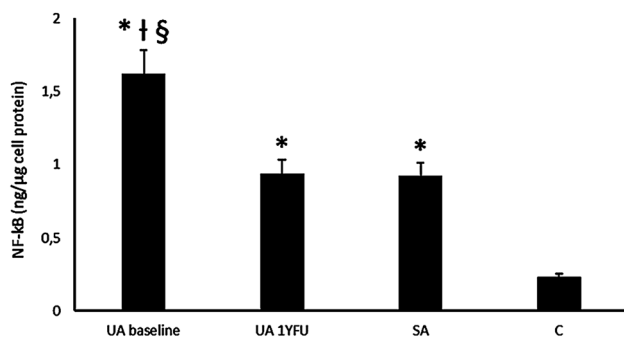
All data (for UA baseline, UA1YFU and SA) are significantly higher compared to C ( $p < 0.001$ ).

Figure 3 shows blood concentrations of IL-6 and IL-1 $\beta$  in UA at baseline, UA1YFU, SA, and C patients. As previously reported [29], IL-6 and IL-1 $\beta$  levels were significantly higher ( $p < 0.001$ ) in UA compared to SA and C. UA1YFU patients show lower levels of IL-6 ( $p < 0.001$ ) compared to UA at baseline, but significantly higher levels compared to SA and C ( $p < 0.001$ ). UA1YFU patients show lower levels of IL-1 $\beta$  ( $p < 0.001$ ) compared to UA at baseline, but no significant differences were found if compared to SA and C.

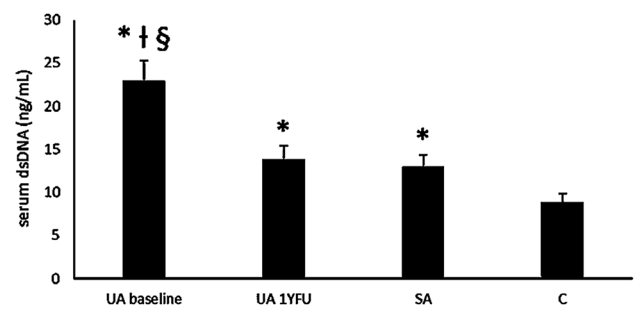
A significant difference ( $p < 0.002$ ) is found in WBC in UA after a 1YFU ( $7.30 \pm 1 \cdot 10^9/L$ ) compared to UA at baseline ( $9.53 \pm 1.8 \cdot 10^9/L$ ). UA1YFU patients have higher WBC levels compared both to SA and C (respectively,  $6 \pm 0.2$  and  $6 \pm 0.1 \cdot 10^9/L$ ;  $p < 0.005$ ).

A significant difference ( $p < 0.001$ ) is found in hs-CRP in UA after a 1YFU,  $0.42$  ( $0.26$ ;  $0.53$ ) mg/dL, compared to UA at baseline,  $0.98$  ( $0.08$ ;  $1.21$ ) mg/dL. No significant differences in hs-CRP levels are found for UA1YFU patients compared to SA and C, respectively,  $0.50$  ( $0.08$ ;  $1$ ) and  $0.43$  ( $0.08$ ;  $0.8$ ) mg/dL.

A significant difference ( $p < 0.001$ ) is found in oxLDL in UA after a 1YFU ( $23.1 \pm 3.54 \mu\text{g/mL}$ ) compared to UA at



**Fig. 1** Concentrations of activated circulating Nuclear Factor kappa B (NF-kB) in unstable angina (UA) patients at baseline and after a 1YFU (UA1YFU), compared to stable angina (SA) patients and control subjects (C). The NF-kB was extracted from peripheral blood mononuclear cells (PBMC) derived from the patients. \* $p < 0.001$  vs C;  $^{\dagger}p < 0.001$  vs SA;  $^{\S}p < 0.001$  vs UA1YFU



**Fig. 2** Serum ds-DNA of unstable angina (UA) patients at baseline and after a 1YFU (UA1YFU), compared to stable angina (SA) patients and control subjects (C). \* $p < 0.001$  vs C;  $^{\dagger}p < 0.001$  vs SA;  $^{\S}p < 0.001$  vs UA1YFU

baseline ( $38.7 \pm 4.39 \mu\text{g/mL}$ ). UA1YFU patients have higher oxLDL levels compared to C ( $12.9 \pm 4 \mu\text{g/mL}$ ;  $p < 0.001$ ), but no significant differences are found compared to SA ( $23.3 \pm 4.4 \mu\text{g/mL}$ ).

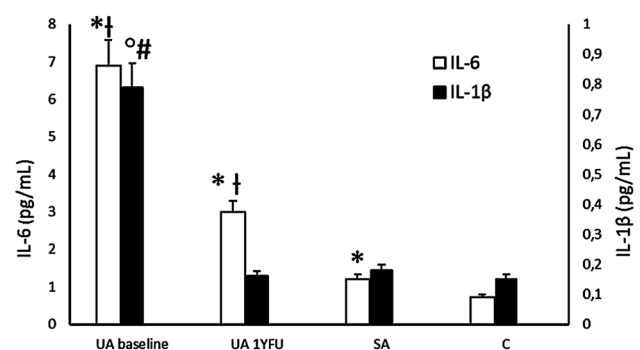
## Discussion

The main finding of this work is that, after a 1YFU, patients with a history of UA improve their inflammatory status, but without achieving the status of C, and becoming comparable to SA subjects.

The focus of this manuscript is to investigate the role of NF-kB in UA patients in their follow-up.

NF-kB has been largely investigated in cardiovascular diseases, in particular in ischemic heart disease [9, 10, 28, 29].

NF-kB is generally well known to worsen cardiac remodelling by activating pro-inflammatory pathways to mediate cardiac hypertrophy and maladaptive remodelling [31, 32].



**Fig. 3** Plasma concentrations of IL-6 and IL-1 $\beta$  in unstable angina (UA) patients at baseline and after a 1YFU (UA1YFU), compared to stable angina (SA) patients and control subjects (C). IL-6: \* $p < 0.001$  vs C;  $^{\dagger}p < 0.001$  vs SA. IL-1 $\beta$ :  $^{\circ}p < 0.001$  vs C;  $^{\#}p < 0.001$  vs UA1YFU and SA

However, the intriguing point concerns the multi-faceted role of NF- $\kappa$ B, in particular its protective role as emerged in different studies mentioned below. This fact opens a debate about the deleterious and protective role of this transcription factor, as recently reviewed [33].

In fact, NF- $\kappa$ B is able to induce the expression of several survival proteins including c-IAP1 and 2, and TRAF-1 and 2 [34–36]. Moreover, NF- $\kappa$ B exerts its protective effects by up-regulating the expression of several genes such as Bcl-2 family members and caspase inhibitors [37].

NF- $\kappa$ B protective action has been shown to be related to the cross-talk with heat shock proteins, normally implicated in the protection against apoptosis [38–40]. The involvement of NF- $\kappa$ B in ischemia re-perfusion injury and protective pathways has been observed [41–44]. In this study, NF- $\kappa$ B levels are significantly lower in UA patients after a 1YFU compared to the year before, and after a 1YFU, they are comparable to SA. The activation of NF- $\kappa$ B in circulating cells of UA patients is, at least in part, induced by oxLDL, as previously demonstrated [28]. The NF- $\kappa$ B persistent activation, also is lower compared to the acute event (with lower levels of oxLDL), can be explained with the double role of this transcription factor, regulating its action according to the different situations.

Inflammation has a well-established role in coronary artery disease [45, 46]. As reported in the previous authors' study [28], the total WBC count and CRP levels are significantly higher in UA patients compared to SA and C. After a 1YFU, UA patients have significantly lower levels of these markers compared to their baseline. This fact is important, because it opens a debate about the usefulness of CRP as a precise follow-up marker. In a prospective multicentre study [47], CRP was measured at admission, at hospital discharge and 1 month later in consecutive patients hospitalized for acute coronary syndrome. This study aimed to determine whether there was a clinical prognostic utility for measuring CRP following an acute coronary episode. In conclusion, the study did not support the clinical use of CRP because of the lack of substantial predictive ability (death, myocardial infarction, or UA) of this marker. Nevertheless, CRP is considered the principal marker able to predict short- and long-term outcome in patients with acute coronary syndrome, with several studies supporting this notion [48–50].

Then, the discussion could be driven toward a particular field of research with the aim of including a proposal of the possible role of NF- $\kappa$ B as mediator in NETs' formation.

According to the results of this study, patients with UA at baseline have significantly higher levels of circulating ds-DNA compared to SA and C. After 1YFU, the UA patients became comparable to SA.

To our knowledge, this is the first study that after a 1YFU compares ds-DNA in UA at baseline to SA and C. Sustained levels of ds-DNA in UA1YFU and SA patients might

indicate that also even without symptoms, these groups of patients are not comparable to healthy subjects. This fact could be revelatory of ongoing tissue damage, but also of the activation of neutrophils in forming NETs. Moreover, UA1YFU patients have higher WBC levels compared both to SA and C. The increased number and the stimulation of neutrophils (represented by ds-DNA) might be driven by NF- $\kappa$ B. In fact, a recent study [51] has shown that NF- $\kappa$ B is involved in the generation of NETs. The authors demonstrate that acetylsalicylic acid (ASA) and NF- $\kappa$ B inhibitors (using two structurally specific inhibitor of I $\kappa$ B $\alpha$  phosphorylation) have an inhibitory effect on NETs formation in vitro. The effects of ASA are not only related to cyclo-oxygenase acetylation, but also include inhibition of NF- $\kappa$ B activation [52]. A previous study [53] shows that the stimulation of neutrophils induces both the nuclear accumulation of NF- $\kappa$ B/Rel proteins and the concomitant degradation of I $\kappa$ B $\alpha$ .

On the basis of these studies, Lapponi et al. [51] conclude that this major inflammatory factor is involved in the inflammatory response mediated by NETs.

Ds-DNA has cytotoxic and pro-thrombotic effects [54], creating a link between inflammation and coagulation. The study by Borissoff [23] clarifies the relationships between extracellular DNA formation, coronary atherosclerosis, and the presence of a pro-thrombotic state. The study reveals that markers of NETosis (ds-DNA, nucleosomes, citrullinated histone H4, and MPO-DNA complexes) are independently associated with the severity of coronary artery disease, a pro-thrombotic state, and also the occurrence of major adverse cardiac events. The study suggests that NETs formation might contribute to atherosclerosis progression.

The previous histological studies have shown the presence of NETs in the luminal portion of mouse and human atherosclerotic lesions [24, 25]. In a very recent study [26], coronary thrombarterectomies derived from patients with ST-elevation acute coronary syndrome (undergoing primary percutaneous coronary intervention) were analyzed. In the culprit lesion site, NETs burden positively correlate with infarct size and negatively with ST-segment resolution. In fact, nucleosomes, ds-DNA, neutrophil elastase, myeloperoxidase, all markers of NETosis are found to be increased in the culprit lesion site [26].

Inflammation contributes to all phases of atherosclerosis [55]. Interleukins, in particular, IL-6 and IL-1 $\beta$ , are critical mediators of the systemic inflammatory response [56, 57].

Secretion of cytokines by inflammatory cells is a major driver of pathogenesis in UA [58]. As previously reported by the authors [29], UA patients exhibit greatly enhanced plasma concentrations of IL-6 and IL-1 $\beta$  compared to SA and C. As discussed [29], this fact might be related to the effect of circulating oxLDL.

Now, after 1YFU, UA patients show lower levels of IL-6 compared to UA at baseline, but significantly higher levels



compared to SA and C. UA1YFU patients show lower levels of IL-1 $\beta$  compared to UA at baseline, but no significant differences are found if compared to SA and C. Thus, the trend of these molecules is quite different.

It is well known that interleukin-1 (IL-1) plays a particularly prominent role in atherothrombosis [59]. In addition, a complex of intracellular proteins, known as the nucleotide-binding leucine-rich repeat-containing pyrin receptor 3 (NLRP3) inflammasome, activates caspase-1 or IL-1 $\beta$  converting enzyme, the protease that produces active IL-1 $\beta$  from its inactive precursor [60–63]. Several exogenous “danger signals” trigger the inflammasome including crystalline compounds. It has been shown that the NLRP3 inflammasome is critical for production of active IL-1 $\beta$  responses not only by bacteria, crystalline uric acid, and crystalline pyrophosphate, but also to cholesterol crystals and minimally modified LDL cholesterol [64].

These facts were the basis of the well-known CANTOS study [65] with Canakinumab.

This fact leads to some considerations, trying to give a link between these cytokines, NETs, and NF- $\kappa$ B regulation.

In a mouse model of atherosclerosis [66], cholesterol crystals act both as priming and danger signals for IL-1 $\beta$  production. Cholesterol crystals trigger neutrophils to release NETs.

In addition, IL-6 is a potent NETs inducer, as previously demonstrated [67]. Nevertheless, this cytokine is principally known for a precise role in atherosclerosis [68]. Large quantities of IL-6 were found in human atherosclerotic plaques [69]. In particular, IL-6 can promote the occurrence of atherosclerosis development and plaque rupture [68, 69].

Different studies [70–72] show that serum IL-6 of acute myocardial infarction patients is significantly higher compared to UA patients. The levels in UA are significantly higher compared to SA. These results agree with the result of the current study. The potential causal role of IL-6 in atherothrombosis has been suggested by its selective expression in macrophages in murine and human atheroma [69, 73, 74]. IL-6 is highly up-regulated at the site of the coronary occlusion. It can be produced by cardiac myocytes under condition of local hypoxia in the viable border zone of re-perfused infarction. [75]. IL-6 is considered a predictor of high-risk coronary anatomy, as defined by coronary computed tomography angiography [76]. At least, circulating IL-6 has been shown to be associated with the thin-cap fibroatheroma, that is the lesion with the highest potential for plaque rupture [77, 78]. All these data suggest that IL-6 levels might correlate with the instability of the atherosclerotic plaque. In fact, IL-6 has a stimulatory effect on smooth muscle cells proliferation [77].

IL-6 is regulated by NF- $\kappa$ B [79, 80]. The hallmark of vascular NF- $\kappa$ B activation is the production of IL-6, whose local role in vascular inflammation has been reviewed [79]. The

ultimate consequence of NF- $\kappa$ B signalling is the activation of inflammatory genes including adhesion molecules and chemotaxins. However, clinically, the hallmark of vascular NF- $\kappa$ B activation is the production of IL-6 [79]. The same trend in UA at baseline, UA1YFU, SA for IL-6, and NF- $\kappa$ B may reflect this fact. In particular, the higher levels in UA1YFU compared to SA might correlate with the plaque instability that led to the UA condition the year before.

All the markers considered in this work are affected by therapy, of course. There is a large knowledge about this fact [26, 29, 45, 50–52, 65]. The small sample of patients in the current study does not allow the exact contribution of each drug in the influence of the final condition. However, the finding that despite *complete* therapy (according to the current guidelines for UA), UA1YFU patients do not reach the condition of C; this fact itself means that the pathology itself makes the difference.

### Study limitations

The main limitation of this study is the lack of complete collection of NETosis markers other than ds-DNA. This fact makes the authors' considerations partially elusive for the moment.

Moreover, several biological and methodological hurdles have been identified in cf-DNA testing, as reviewed [81] (different testing methods, great variability of the levels in the healthy population, etc.). Nevertheless, up to now, no precise data are available about ds-DNA. It is reasonable that similar considerations could also be done for these DNA fragments.

The absence of a follow-up sampling in the SA and C is a further consistent limitation of the study.

However, the strength of the study is underlining the new overview of the role of NF- $\kappa$ B, as a protective factor connected with NETosis in UA. These notions must be elevated to a new degree when considering the enormously complicated interacting networks that explain the complex and not fully investigated mechanisms that link immunity, inflammation, and cardiovascular diseases. There are very few data in the literature about the hypothesis that NF- $\kappa$ B may act as the mediator of NETs' formation. A very recent paper, in a different context (Dermatology and Wound healing), analyzes this fact. NETs' scaffold recognized by Toll-Like Receptor 9 (TLR 9) is able to activate the NF- $\kappa$ B pathway. NETs' stimulation rapidly induces a dose dependent NF- $\kappa$ B activation and such signalling pathway modulates keratinocytes proliferation [82].

### Conclusions

After a 1YFU, patients with a history of UA improve their inflammatory status, but without achieving the status of C, and becoming comparable to SA subjects.

In conclusion, the persistent activation of NF- $\kappa$ B in these patients might also be considered a conceivable solution to maintain an innate immunity response, as NETosis is. NF- $\kappa$ B activation and NETs formation are, therefore, similar to a double-edged sword, acting not only as an effective first-line defence mechanism, but also leading to organ failure and death if the process is uncontrolled.

**Author contributions** CM and LC conceived the study; GP statistically analyzed the data; AF and UG revised the data and the manuscript; GS and CS performed the experiments; CM wrote the manuscript.

## Compliance with ethical standards

**Conflict of interest** The Authors declare that they have no conflict of interest.

**Statement of human and animal rights** The study was conducted in accordance with the ethical standards laid down in the Helsinki Declaration of 1975 and its late amendments.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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